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Biopolymer particles for proteins and peptides sustained release produced by supercritical emulsion extraction

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Abstract

In this work, biodegradable polymers have been successfully used to encapsulate proteins and peptides to prolong their therapeutic effect. Biodegradable microspheres prepared using poly(lactic acid), PLA, and its copolymers with poly(glycolic acid), PLGA, have been used as protein delivery systems. To overcome common limitation of the conventional emulsion/solvent removal techniques SC-CO2 has been proposed as extracting agent of the ‘‘oily’’ phase of emulsions, to lead to solvent-free microparticles. Supercritical emulsion extraction, produces an aqueous suspension of microparticles after the elimination of the organic solvent contained in the emulsion micelles. The process is very fast, due to the enhanced mass transfer of SC-CO2; the fast extraction rate results in a narrower particle size distribution (PSD) because droplets aggregation is minimized. The new proposed layout produces micro and submicrospheres in a robust and reproducible mode. The continuous process enhances the mass transfer due to a large contact area between SC-CO2 and emulsions in the tower, allowing the production of microspheres in short processing times and a higher throughput with smaller plant volumes eliminating the batch-to-batch repeatability problems. Biopolymer nanoparticles in the range 200-400 nm and microparticles in the range 1-4 μm encapsulating bovine serum albumin (BSA) as model protein and insulin like growth factor (h-IGF) were produced. The solvent of the oil phase was completely removed operating at 37°C and 100 bar, preventing proteins from degradation.

Keywords: Supercritical Emulsion Extraction, microparticles, nanoparticles, proteins

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1. Introduction

Tissue engineering and regenerative medicine hold great promise to restore tissue and organ functions with the ambitious goal to avoid organ transplantation. In vitro and in vivo tissue engineering strategies generally include signalling molecules, which should try to reproduce the natural sequence of signals guiding spontaneous tissue repair. Signalling molecules are mainly growth factors (GFs), a wide variety of proteins with distinct properties which are able to act on cell migration, differentiation, proliferation and organization in a functional tissue [1].

Proteins possess small half-lives and are not capable to diffuse through biological membranes. Their instability in the stomach and intestine makes oral delivery of these drugs difficult. Alternative administration by frequent injections is also tedious and expensive. Therefore, the development of biodegradable polymeric microspheres has been seen as a promising way to overcome the administering problems of these macromolecules [2]. A vehicle is needed capable of encapsulating proteins, minimizing the mechanisms of degradation and maximizing the in vivo activity, and providing controlled release that can be delivered via parental administration is needed.

Proteins and GFs controlled release from particulate devices has been widely reported in the literature [3]. Frequently, these delivery systems were based on natural polymers, such as gelatin and alginate [4,5]. Particulate systems based on these polymers are able to encapsulate GFs with minimum loss of bioactivity of the encapsulated molecule. However, they lack the capacity to provide long-term release, particularly when formed as nanoparticles. These limitations can be overcome by particulate systems based on biodegradable polyesters (i.e., PLA, PLGA) [6].

However, few sustained release therapeutic protein products based on biodegradable polymers have received FDA approval due to the inherent instability of proteins when they are exposed to the conditions normally encountered during microparticle fabrication using conventional techniques. Other problems encountered are low encapsulation efficiencies and high burst release of the drug. For many therapeutic proteins achieving an acceptable dose volume (i.e., high drug content) while maintaining satisfactory release kinetics (i.e., minimal burst, acceptable duration) represents a very significant formulation challenge [7].

Different conventional methods have been reported for the preparation of micro and nanoparticles including dispersion polymerization [8], nanoprecipitation [9], solvent evaporation of emulsions [10], spray drying [11]. These techniques show some drawbacks such as, the use of toxic solvents that have to be removed [12,13], and low encapsulation efficiency [14,15]. Conventional solvent evaporation usually proceeds at a slow rate and may also require high temperature or reduced pressure to eliminate the organic solvent; solvent extraction requires a large amount of external aqueous phase to extract completely the solvent [16,17]. Moreover, it is difficult to obtain monodisperse particles using this conventional emulsion based technique because, generally, polydisperse microspheres are obtained due to the fusion or coagulation of emulsion droplets under mechanically agitation during evaporation [18].

A different approach, using a new supercritical fluid technology was recently proposed; SuperCritical-Carbon Dioxide (SC-CO_2) is used as the extracting agent of the oily phase of emulsions to produce solvent-free micro or nanoparticles [17,19,20]. Using this new technology named Supercritical Emulsion Extraction (SEE) and developed in a continuous layout (SEE-C), the dimension of the biopolymer particles are directly related to the dimension of the droplets in the emulsions by a specific shrinking factor, related to polymer concentration in the oily phase [21]. The continuous operating layout is obtained by means of high-pressure packed tower for emulsion/SC-CO_2 contact in counter-current mode, allowing a robust and reproducible production [22]. The SEE-C technology has a great potential regarding the field of protein encapsulation thanks to the mild extraction condition and the short process time.

For these reasons, the objectives of this work is to demonstrate that it is possible to apply the SEE-C to the production of biopolymeric micro and nanoparticles encapsulating peptides and proteins starting from double emulsion. Bovine serum albumin (BSA) has been selected as model protein while insulin like growth factor (h-IGF) has been used as peptide.

2. Materials & Methods

2.1. Materials

CO2 (99.9%, SON, Naples, Italy), polyvinyl alcohol (PVA, MW: 30.000–55.000, Aldrich Chemical Co.), polysorbate (Tween 80, Aldrich Chemical Co.), poly-lactic acid (PLA, MW: 28.000, Resomer R 203H, Boehringer), PLGA (PLGA, 75:25 MW: 20000, Resomer RG 752S, Boehringer), ethyl acetate (EA, purity 99.9%, Aldrich Chemical Co.) were used as received. Bovine pancreas albumin (BSA, purity 99.9%) was obtained by Sigma-Aldrich Co. (Milan, Italy). h-IGF was supplied from PeproTech.

2.2. Emulsion preparation

w-o-w emulsions for the encapsulation of BSA (w-o-w ratio: 1:19:80 w/o/w) were prepared using 1mL of water/PVA solution (0.04% w/w of PVA), as internal water phase, in which BSA was dispersed at 0.5% w/w, that was added into polymer/EA solutions (1-10% w/w of PLGA or PLA) and sonicated using the Digital Sonifier Branson (mod. 450, ½" diameter micro-tip, 20 kHz). The primary w-o emulsion was, then, added into water Tween80 (0.6% w/w Tween80) solution to form the secondary emulsion using a high-speed stirrer (mod. L4RT, Silverson Machines Ltd., Waterside, Chesham Bucks, UK). w-o-w emulsions for the encapsulation of h-IGF were produced following the same procedure described above, adding in the water internal phase the h-IGF at a concentration of 5μg/mL.

2.3. SEE-C apparatus description

Detailed description of the SEE-C apparatus is reported in Fig 1**.** It consisted of a 100 cm long column with an internal diameter of about 1 cm. The high pressure column included three stages formed of stainless steel cylindrical elements of 30 cm height connected by four way cross- unions and packed with stainless steel packing. The apparatus was thermally insulated by ceramic cloths and its temperature profile was controlled by six temperature controllers; cross-unions were also used to insert temperature controls at different heights of the column. SC-CO_2 was fed at the bottom of the column by a high-pressure diaphragm pump (model Milroyal B; Milton Roy, Pont Saint-Pierre, France) at a constant flow rate. The emulsion was taken from a reservoir and fed to the column by a high pressure piston pump (model 305; Gilson, Villiers le Bel, France) at the top of the column at a constant flow rate. A separator, located downstream the top of the column, was used to recover the extracted oily phase. Particles suspension was continuously collected at the bottom of the column by decompression using a needle valve. At the end of each run, the suspension was washed several times by centrifugation at 6500 rpm for 45 minutes with distilled water, recovered by membrane filtration (porosity: 0.1 or 0.4 μm) and lyophilized.

2.4. Analytical methods

Field Emission-Scanning Electron Microscope (FE-SEM, mod. LEO 1525; Carl Zeiss SMT AG, Oberkochen, Germany) was used to study the morphology of the produced microspheres. A sample of powder was dispersed on a carbon tab previously stuck to an aluminum stub. Samples were coated with gold (layer thickness 250Å) using a sputter coater (mod.108 A, Agar Scientific, Stansted, UK). Droplet size distributions (DSD) and particle size distributions (PSD) of microparticles were measured by dynamic light scattering (DLS, mod. Mastersizer S, Malvern Instruments Ltd., Worcherstershire, UK). PSD and DSD of nanoparticles were measured by DLS using a Malver Zeta Sizer instrument (mod. Zetasizer Nano S, Worcestershire, UK).

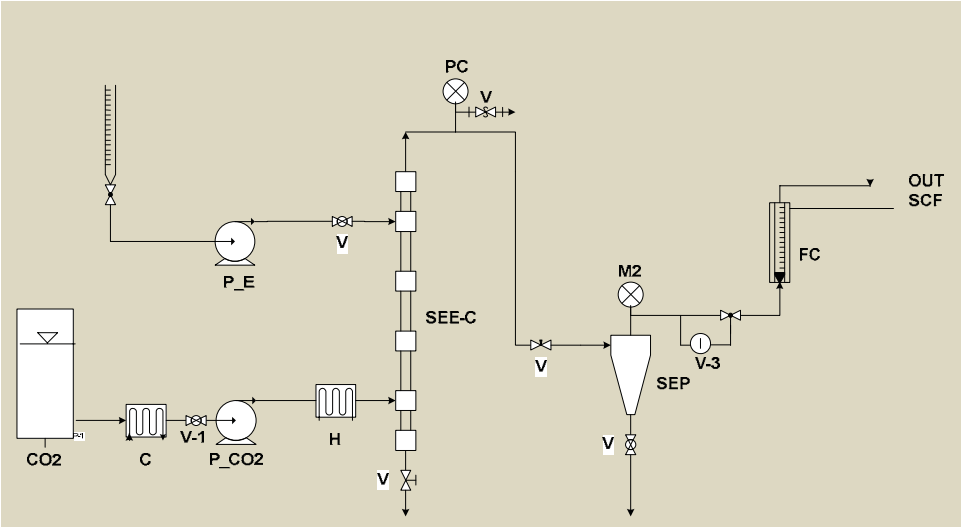

Fig. 1. Detailed description of the SEE-C apparatus layout. Vessel CO_2 (CO_2); Cooler (C); Pump_CO_2 (P_CO2); heater (H); Pump_Emulsion (P_E); on-off valves (V); micrometering valve, (V-3); Manometer (PC); Flow measurement (FC); Separator (SEP).

Extraction pressure and temperature conditions fixed at 80 bar and 38°C were used to process emulsions with an oily phase content of about 20% w/w. They were selected to assure the complete miscibility of EA in SC-CO_2 [21]; a temperature of 38°C is also compatible with protein processing. The counter-current operation in the packed column is also favored by large density differences between the two phases involved in the process. Using supercritical fluid, the lower is the pressure the larger is the density difference between a liquid and SC-CO_2 at fixed temperature. Therefore, fixing the temperature at 38°C and operating at 80 bar, the maximum difference in density between the emulsion and SC-CO_2 is obtained because they are of 1 g/cm^3 against 0.3 g/cm^3, respectively. Finally, the ratio between the emulsion and SC-CO_2 (liquid/gas ratio, L/G) was fixed at 0.1 according to previous optimization [21], to obtain the minimum solvent residue in the final suspension.

3. Results and discussion

3.1. BSA encapsulation study

Micro and nanoparticles of PLGA and PLA loaded with BSA were produced using a double emulsion w/o/w (1/19/80) with a water internal phase charged with BSA (2.5-5 mg/mL) and PVA (0.04% w/w).

In the case of microparticle production, the oily dispersed phase was formed by a polymer solution in ethyl acetate at 10% w/w. The water external phase was formed by water saturated with ethylacetate plus Tween80 as surfactant (0.6% w/w). The primary emulsion w/o was obtained by ultrasonication. Then the primary emulsion was added to the external water by a rotative system at 3300 rpm for 6 minutes. Optical images of emulsions obtained using PLGA and PLA and at different BSA loading are reported in the figures 2 a-c. FESEM images of the microparticles obtained after SEE-C process are also reported in figures 2d-f.

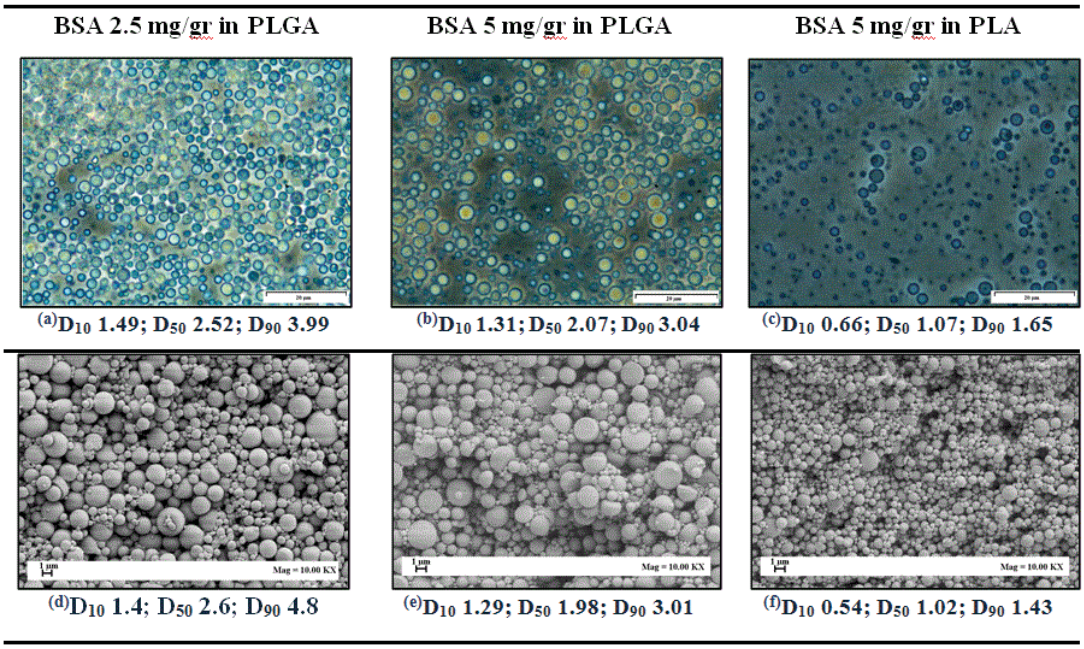

Fig. 2. a-f. Optical microscope images of different emulsion obtained for microparticles production. Two different polymer PLGA and PLA are used at two different BSA loading. FESEM images of the microsphere produced after SEE-C are also reported.

Images in Fig. 2. show that stable emulsions were formed in each case with well defined droplets. Polymer precipitation inside the droplets was induced by SC-CO_2 extraction of the organic solvent of the oily dispersed phase and microparticles suspension was obtained with a yield higher than 90%. Particles were always spherical and non coalescing. A medium shrinkage factor of 4% between droplet mean size of the emulsion and particles mean size was observed. In particular PLGA gives particles with MS =2.6 and SD=0.56 μm, while PLA, at the same process conditions, gives particles with MS =1.98 and SD=0.28 μm. BSA was encapsulated with two different loading 2.5 mg/gr and 5 mg/gr of PLGA and no considerable variation in the PSD of the microsphere obtained was observed with respect of the two different loading tested.

In the case of nanoparticle production, the oily phase was formed by a polymer solution in ethyl acetate at 1% w/w. The primary w/o emulsion was obtained again by ultrasonication. Then, it was added to the external water phase by a rotative system operating at 6000 rpm for 6 minutes. At the end the final emulsion was sonicated again at 30% amplitude for 2 minutes. Examples of FE-SEM images of nanoparticles of PLGA and PLA with BSA loading of 5 mg/gr of polymer obtained after SEE-C processing are reported in Fig. 3.a-c, respectively. DSD of the emulsions and PSD of the suspensions were also reported for comparison in Figures 3b-d.

Nanoparticles with a mean diameter of about 300 nm were produced in both cases after SEE process, PLA gives more uniform PSD with a smaller SD. A medium shrinkage factor of 15% between droplet mean size of the emulsion and particles mean size of the suspension of nanoparticles was observed.

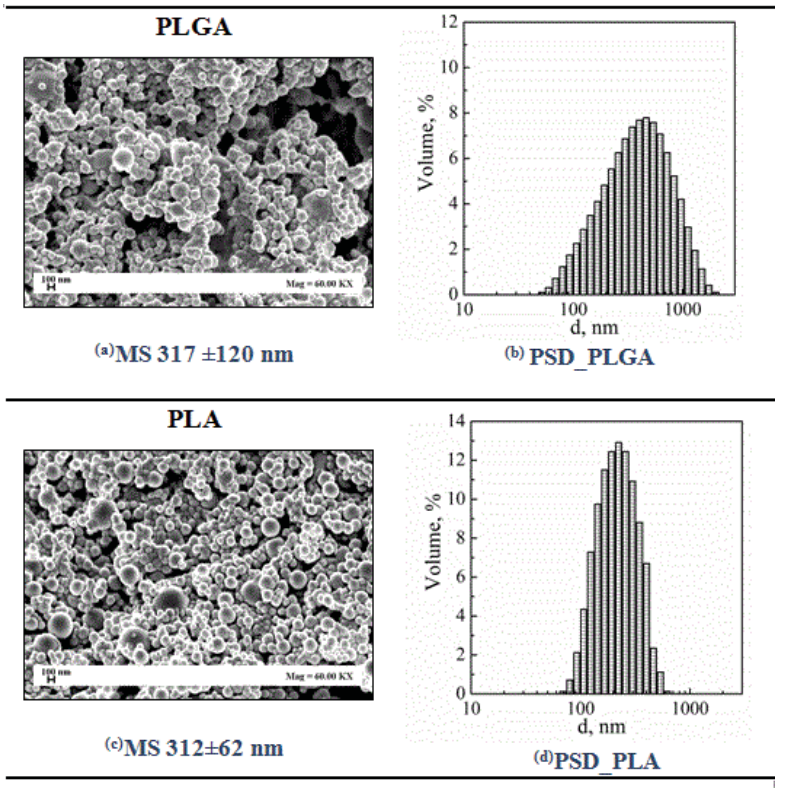

Fig. 3. a d. FESEM images of the nanosphere produced after SEE-C process are also reported. DSD and PSD are also reported.

3.2. h-IGFs encapsulation

Microparticles of PLGA loaded with h-IGF were produced using the same emulsion described in the case of BSA encapsulation in PLGA microparticles; a double emulsion w/o/w (1/19/80) was used in which in the internal water phase at the aqueous solution of BSA (2.5 mg/mL) was added 5 ug/mL of h-IGF and PVA (0.04% w/w). BSA also acts as stabilizing and protecting agent for the growth factor. The oil phase was composed of a polymeric solution in ethyl acetate at 10% w/w of PLGA. The water external

phase was formed by water saturated with ethylacetate plus Tween80 as surfactant (0.6% w/w). The primary emulsion w/o was obtained again by ultrasonication. Then the primary emulsion was added to the external water phase and the emulsion was obtained agitating the system at 3300 rpm for 6 minutes.

An optical image of the produced emulsions and a SEM image of the derived devices are reported in Fig. 4a-b. Again produced PLGA microparticles were spherical and non coalescing. Microparticles with a mean size of 2.5 μm (SD=0.3) μm were obtained. The PSD was the same of the one obtained using the same system but charged only with BSA and described before, confirming the high reproducibility of the SEE-C process. Loading measurement and release profiles are in progress.

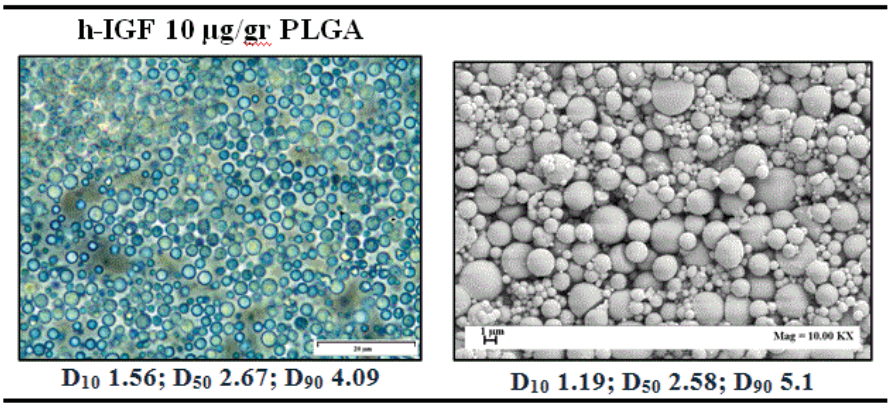

Fig. 4. a-b. Optical microscope images of emulsion obtained for the production of PLGA microparticles encapsulating h-IGF. FE-SEM images of the microsphere produced after SEE-C process are also reported.

4. Conclusions

SEE was successfully applied for the production of micro and nanospheres of PLGA and PLA encapsulating a protein and a peptide. Different emulsion formulation and preparation methods were applied tuning particles dimensions form 2.5 μm to 300 nm for both polymer tested. The same emulsion formulation used to produce microparticles of PLGA was used to encapsulate h-IGF. Emulsions produced were always stable and non coalescing. Precipitation of PLGA or PLA inside the droplets is induced by SC-CO_2 extraction of the organic solvent of the oily dispersed phase using a continuous counter-current tower. Thanks to the peculiar properties of the SC-CO_2, opportunely chosen the operative conditions of the SEE-C process in terms of pressure, temperature and liquid and gas flow rate, a very efficient and fast extraction of the oily solvent of the emulsion is obtained, producing the hardening of the polymer and the entrapment of the protein/peptide inside the particles. PSD very close to DSD were obtained demonstrating the absence of aggregation phenomena during the SEE-C process.

Further study of characterization of the SEE-C products obtained and illustrated in this work are in progress. In particular protein and peptides loading and release are under study, as well as investigation about the possible degradation of the proteins/peptides during the SEE-C process.

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